

TITLE OF THE INVENTION
SYNTHETIC HUMAN PAPILLOMAVIRUS GENES

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5 FIELD OF THE INVENTION

This invention relates to human papillomavirus (HPV) genes which have been codon-optimized for expression in a human cellular environment, and their use with adenoviral vectors and/or plasmid vectors as vaccines.

10 BACKGROUND OF THE INVENTION

Papillomavirus infections occur in a variety of animals, including humans, sheep, dogs, cats, rabbits, snakes, monkeys and cows. Papillomaviruses infect epithelial cells, generally inducing benign epithelial or fibroepithelial tumors at the site of infection. Papillomaviruses are species specific infective agents; a human papillomavirus cannot infect a non-human.

15 Papillomaviruses are small (50-60nm), nonenveloped, icosahedral DNA viruses that encode up to eight early and two late genes. The open reading frames (ORFs) of the virus are designated E1 to E7 and L1 and L2, where "E" denotes early and "L" denotes late. L1 and L2 code for virus capsid proteins. The early genes
20 are associated with functions such as viral replication and cellular transformation.

In humans, different HPV types cause distinct diseases, ranging from benign warts (for examples HPV types 1, 2, 3) to highly invasive genital and anal carcinomas (HPV types 16 and 18). At present there is not a satisfactory therapeutic regimen for these diseases.

25 Immunological studies in animals have shown that the production of neutralizing antibodies to papillomavirus antigens prevents infection with the homologous virus. However, development of a vaccine has been hindered by the difficulties associated with culture of the papillomavirus *in vitro*.

Vaccination is an effective form of disease prevention and has proven
30 successful against several types of viral infection. However, to date, attempts to generate an effective HPV vaccine have not been entirely successful.

SUMMARY OF THE INVENTION

This invention relates to oligonucleotides which encode a human papillomavirus (HPV) protein which has been codon-optimized for efficient expression in a host cell; preferably the oligonucleotides are DNA. In one embodiment, the polynucleotides encode a protein which retains its wild-type amino acid sequence. In an alternate embodiment, the polynucleotides encode a mutated form of a HPV protein which has reduced protein function as compared to wild-type protein, but which maintains immunogenicity. This invention also relates to the mutated HPV proteins so encoded.

In preferred embodiments, the protein is selected from the group consisting of: L1, L2, E1, E2, E4, E5, E6 and E7 proteins. Particularly preferred are L1, L2, E2, and E7 proteins.

Another aspect of this invention is a vector carrying the polynucleotides encoding a codon-optimized HPV protein. Yet another aspect of this invention are host cells containing these vectors.

In a preferred embodiment, the vector is an adenoviral vector. In a particularly preferred embodiment, the adenoviral vector is a vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

- a) a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins or mutant forms thereof, wherein the polynucleotide is codon-optimized for expression in a human host cell; and
- b) a promoter operably linked to the polynucleotide.

Another type of vector which is envisioned by this invention is a shuttle plasmid vector comprising a plasmid portion and an adenoviral portion, the adenoviral portion comprising: an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

- a) a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein the polynucleotide is codon-optimized for expression in a human host cell; and
- b) a promoter operably linked to the polynucleotide.

This invention also is directed to plasmid vaccine vectors, which comprise a plasmid portion and an expressible cassette comprising

a) a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein the polynucleotide is codon-optimized for expression in a human host cell; and

b) a promoter operably linked to the polynucleotide.

5 This invention also relates to vaccine compositions comprising a vector which carries the oligonucleotides to a human host, and allows for expression of the encoded protein. The protein is expressed in an amount sufficient to induce an immune response. In preferred embodiments, the vector is a plasmid vector or an adenoviral vector.

10 This invention also relates to a method of making a HPV protein comprising expressing in a host cell a synthetic polynucleotide encoding a human papillomavirus (HPV) protein, or mutated form of a HPV protein which has reduced protein function as compared to wild-type protein, but which maintains immunogenicity, the polynucleotide sequence comprising codons optimized for expression in
15 a human host.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is the nucleotide sequence of a codon-optimized HPV16 L1 gene (SEQ.ID.NO:1).

20 FIGURE 2 is the nucleotide sequence of a codon-optimized HPV16 E1 gene (SEQ.ID.NO:2). In this particular sequence, there are further mutations which changes the amino acid sequence of the expressed protein-- the glycine residue at position 428 has been converted to aspartic acid, and the tryptophan residue at position 439 is now arginine.

25 FIGURE 3 is the nucleotide sequence of a codon-optimized HPV16 E2 gene (SEQ.ID.NO:3). In this particular sequence, the glutamic acid residue at position 39 has been changed to an alanine, and the isoleucine residue at position 73 has also been changed to an alanine.

30 FIGURE 4 is the nucleotide sequence of a codon-optimized HPV16 E7 gene.(SEQ.ID.NO:4). In this particular sequence, the cysteine residue at position 24 has been changed to glycine, and the glutamic acid residue at position 26 has been changed to a glycine.

FIGURE 5 is the nucleotide sequence of a codon-optimized HPV6a E7 gene (SEQ.ID.NO:5).

FIGURE 6 is the nucleotide sequence of a codon-optimized HPV18 E7 gene (SEQ.ID.NO:6).

FIGURE 7 is the nucleotide sequence of a codon-optimized HPV6a E2 gene (SEQ.ID.NO:7).

5 FIGURE 8 is the nucleotide sequence of a codon-optimized HPV18 E2 gene (SEQ.ID.NO:8).

FIGURE 9 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with native (lanes d, e, f) or synthetic (a, b, c) HPV16 L1 sequences in the expression vector V1Jns.

10 FIGURE 10 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with native or synthetic HPV16 E1 sequences in the expression vector V1Jns. Lanes a and d contain native HPV16 E1 sequences; lanes b and e contain synthetic HPV16 E1, and lane c is a mock-transfected control.

15 FIGURE 11 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with native or synthetic HPV16 E2 sequences in the expression vector V1Jns. Lane a is mock-infected; lane b is lacZ control; lane c contains a synthetic HPV16 E2 isolate #6; lane d contains synthetic HPV16 E2 isolate #11, and lane e has native HPV16 E2.

20 FIGURE 12 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with native or synthetic HPV16 E7 sequences in the expression vector V1Jns. Lane a is mock-infected; lane b is lacZ control; lane c contains synthetic HPV16 E7 isolate #2; lane d is synthetic HPV16 E7 isolate 4; and lane e is native HPV16 E7.

25 FIGURE 13 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with synthetic HPV6a E7 sequences in the expression vector V1Jns. Lanes b and c contain synthetic HPV6a E7 sequences; lane d contains a lacZ control, and lane a is a mock-transfected control.

30 FIGURE 14 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with synthetic HPV18 E7 sequences in the expression vector V1Jns. Lanes b, c, d, and e contain synthetic HPV18 E7 sequences; lane f contains synthetic HPV16 E7 as an antibody control, and lane a is a mock-transfected control.

FIGURE 15 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with synthetic HPV6a E2 sequences in the expression vector VIJns. Lanes a and b contain synthetic E2 sequences; lane c is a beta-gal control, and lane d is mock-transfected.

- 5 FIGURE 16 shows the results of immunoblot analysis of lysates of 293 cells transiently-transfected with synthetic HPV18 E2 sequences in the expression vector VIJns. Lane a is beta-gal control; lane b is mock transfected; and lanes c and d have synthetic sequences.

- 10 FIGURE 17 is a table of oligonucleotides (SEQ.ID.NOS:9-32) used to generate synthetic HPV16 L1.

FIGURE 18 is a table of oligonucleotides (SEQ.ID.NOS:33-64) used to generate synthetic HPV16 E1.

FIGURE 19 is a table of oligonucleotides (SEQ.ID.NOS:65-84) used to generate synthetic HPV16 E2.

- 15 FIGURE 20 is a table of oligonucleotides (SEQ.ID.NOS:85-90) used to generate synthetic HPV16 E7.

FIGURE 21 is a table of oligonucleotides (SEQ.ID.NOS:91-96) used to generate synthetic HPV6a E7.

- 20 FIGURE 22 is a table of oligonucleotides (SEQ.ID.NOS:97-102) used to generate synthetic HPV18 E7.

FIGURE 23 is a table of oligonucleotides (SEQ.ID.NOS:103-126) used to generate synthetic HPV6a E2.

FIGURE 24 is a table of oligonucleotides (SEQ.ID.NOS:127-150) used to generate synthetic HPV18 E2.

- 25 FIGURE 25 is a Western blot of JCL-031 cell lysate. Cell lysate was prepared from JCL-031 cells grown in selection medium containing 400 μ g/mL G418. The immunoblot was developed with anti-HPV 16 E2 (goat 248) antisera. Positions of molecular weight markers are indicated.

- 30 FIGURE 26 shows protection from JCL-031 cell-induced tumor outgrowth. E2 DNA- or control DNA-immunized mice were challenged by subcutaneous injection of 5×10^5 JCL-031 cells into the left inguinal region. Beginning five days after this challenge, all animals were observed at two or three

day intervals until four weeks after inoculation. Tumors were detected and monitored by visual inspection, palpation of the inguinal region, and measurement of tumor diameter with calipers.

5 The term "promoter" as used herein refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers".

10 The term "cassette" refers to the sequence of the present invention which contains the nucleic acid sequence which is to be expressed. The cassette is similar in concept to a cassette tape; each cassette has its own sequence. Thus by interchanging the cassette, the vector will express a different sequence. Because of the restrictions sites at the 5' and 3' ends, the cassette can be easily inserted, removed
15 or replaced with another cassette.

 The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

 The term "effective amount" means sufficient vaccine composition is
20 introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

 "Synthetic" means that the HPV gene has been modified so that it contains codons which are preferred for human expression. In many cases, the amino acids encoded by the gene remain the same. In some embodiments, the synthetic
25 gene may encode a modified protein.

 The term "native" means that the gene contains the DNA sequence as found in occurring in nature. It is a wild type sequence of viral origin.

DETAILED DESCRIPTION OF THE INVENTION

30 Synthetic DNA molecules encoding various HPV proteins are provided. The codons of the synthetic molecules are designed so as to use the codons preferred by the projected host cell, which is preferred embodiments is a human cell. The synthetic molecules may be used as a polynucleotide vaccine which provides effective immunoprophylaxis against papillomavirus infection through neutralizing

antibody and cell-mediated immunity. The synthetic molecules may be used as an immunogenic composition. This invention provides polynucleotides which, when directly introduced into a vertebrate *in vivo*, including mammals such as primates and humans, induce the expression of encoded proteins within the animal.

- 5 The gene encoding a L1, E1, E2 and/or E7 from any serotype HPV can be modified in accordance with this invention. It is preferred that the HPV chosen be one which is known to cause a pathological condition in humans. For this reason, it is preferred that the HPV gene be selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45,
10 HPV51, HPV52, HPV56, HPV58, HPV68 or variants thereof. The vaccine formulation of this invention may contain a mixture of HPV type protein genes (for example, genes from HPV6, 11, 16 and 18), and/or it may also contain a mixture of protein genes (i.e. L1, E1, E2, and/or E7).

15 Codon optimization

The wild-type sequences for many HPV genes are known. In accordance with this invention, HPV gene segments were converted to sequences having identical translated sequences but with alternative codon usage as defined by Lathe, 1985 "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations" *J. Molec. Biol.* 183:1-12, which is hereby incorporated by reference. The methodology may be summarized as follows:

1. Identify placement of codons for proper open reading frame.
2. Compare wild type codon for observed frequency of use by human genes.
- 25 3. If codon is not the most commonly employed, replace it with an optimal codon for high expression in human cells.
4. Repeat this procedure until the entire gene segment has been replaced.
5. Inspect new gene sequence for undesired sequences generated
30 by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.) and substitute codons that eliminate these sequences.

6. Assemble synthetic gene segments and test for improved expression.

In accordance with this invention, it has been found that the use of alternative codons encoding the same protein sequence may remove the constraints on expression of HPV proteins by human cells.

These methods were used to create the following synthetic gene segments for various papillomavirus genes creating a gene comprised entirely of codons optimized for high level expression. While the above procedure provides a summary of our methodology for designing codon-optimized genes for DNA vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence.

In some embodiments of this invention, alterations have been made (particularly in the E-protein native protein sequences) to reduce or eliminate protein function while preserving immunogenicity. Mutations which decrease enzymatic function are known. Certain alterations were made for purposes of expanding safety margins and/or improving expression yield. These modifications are accomplished by a change in the codon selected to one that is more highly expressed in mammalian cells. In the case of HPV16 E1, for example two mutations were introduced: glycine at amino acid number 482 was changed to aspartic acid by conversion of GGC to GAC; and tryptophan was changed to arginine at position 439 by conversion of TGG to CGC.

For HPV16 E2, conversion of glutamic acid at position 39 to alanine and isoleucine at position 73 to alanine by conversion of both codons each to GCC.

For HPV16 E7, conversion of cysteine at position 24 to glycine and glutamic acid at position 26 to glycine was permitted by alteration of TGC and the GAG respectively both to GGC.

The codon-optimized genes are then assembled into an expression cassette which comprises sequences designed to provide for efficient expression of the protein in a human cell. The cassette preferably contains the codon-optimized gene, with related transcriptional and translations control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the cytomegalovirus promoter with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a



number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the bovine growth hormone terminator, although other known transcriptional terminators may also be used. The combination of CMVintA-BGH terminator is particularly

5 preferred.

Examples of preferred gene sequences are given in SEQ.ID.NOS: 1-8.

VECTORS

In accordance with this invention, the expression cassette encoding at
10 least one HPV protein is then inserted into a vector. The vector is preferably a plasmid or an adenoviral vector, although linear DNA linked to a promoter, or other vectors, such as adeno-associated virus or a modified vaccinia virus vector may also be used.

If the vector chosen is an adenovirus, it is preferred that the vector be a
15 so-called first-generation adenoviral vector. These adenoviral vectors are characterized by having a non-functional E1 gene region, and preferably a deleted adenoviral E1 gene region. In some embodiments, the expression cassette is inserted in the position where the adenoviral E1 gene is normally located. In addition, these vectors optionally have a non-functional or deleted E3 region. The adenoviruses can
20 be multiplied in known cell lines which express the viral E1 gene, such as 293 cells, or PerC.6 cells.

For convenience in manipulating the adenoviral vector, the adenovirus
may be in a shuttle plasmid form. This invention is also directed to a shuttle plasmid
vector which comprises a plasmid portion and an adenovirus portion, the adenovirus
25 portion comprising an adenoviral genome which has a deleted E1 and optional E3 deletion, and has an inserted expression cassette comprising at least one codon-optimized HPV gene. In preferred embodiments, there is a restriction site flanking the adenoviral portion of the plasmid so that the adenoviral vector can easily be removed. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic
30 cells.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the adenoviruses, shuttle plasmids and DNA immunogens of this invention.

If the vector chosen is plasmid DNA, it is preferred that the vector contain one or more promoters recognized by mammalian or insect cells. In a preferred embodiment, the plasmid would contain a strong promoter such as, but not limited to the CMV promoter. The gene to be expressed would be linked to such a promoter. An example of such a plasmid would be the mammalian expression plasmid VIJns as described (J. Shiver et. al. 1996, in *DNA Vaccines*, eds., M. Liu, et al. N.Y. Acad. Sci., N.Y., 772:198-208 and is herein incorporated by reference).

In some embodiment of this invention, the both the vaccine plasmid and the adenoviral vectors may be administered to a vertebrate in order to induce an immune response. In this case, the two vectors are administered in a "prime and boost" regimen. For example the first type of vector is administered, then after a predetermined amount of time, for example, 1 month, 2 months, six months, or other appropriate interval, a second type of vector is administered. Preferably the vectors carry expression cassettes encoding the same polynucleotide or combination of polynucleotides.

Thus, another aspect of this invention is a method for inducing an immune response against human papillomavirus in a vertebrate, comprising

- A) introducing into the vertebrate a first vector comprising a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein the polynucleotide is codon-optimized for expression in a human host cell;
- B) allowing a predetermined amount of time to pass; and
- C) introducing into the vertebrate a second vector comprising adenoviral vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprises
 - i) a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins or mutant forms thereof, wherein the polynucleotide is codon-optimized for expression in a human host cell; and
 - ii) a promoter operably linked to the polynucleotide.

In general, is preferred that the first vector be a plasmid vaccine vector and the second vector be an adenoviral vector. Thus this invention is directed to a method for inducing immune responses in a vertebrate comprising:

A) introducing into the vertebrate a plasmid vaccine, wherein the plasmid vaccine comprises a plasmid portion and an expression cassette portion, the expression cassette portion comprising:

5 i) a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein the polynucleotide is codon-optimized for expression in a human host cell; and

ii) a promoter operably linked to the polynucleotide;

B) allowing a predetermined amount of time to pass; and

10 C) introducing into the vertebrate an adenoviral vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

i) a polynucleotide encoding an HPV protein selected from the group consisting of L1, E1, E2, and E7 proteins or mutant forms thereof, wherein the polynucleotide is codon-optimized for expression in a human host cell; and

15 ii) a promoter operably linked to the polynucleotide.

In yet another embodiment of the invention, the codon-optimized genes may be introduced into a recipient by way of a plasmid or adenoviral vector, as a "prime", and then a "boost" is accomplished by introducing into the recipient a polypeptide or protein which is essentially the same as that which is encoded by the
20 codon-optimized gene. Fragments of a full length protein may be substituted, especially those which are immunogenic and/or include an epitope.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend partially on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene
25 product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 μ g to 300 μ g of a plasmid vaccine vector is administered directly into muscle tissue. An effective dose for recombinant adenovirus is approximately 10⁶-10¹² particles and preferably about 10⁷-10¹¹ particles. Subcutaneous injection, intradermal introduction, impression through the
30 skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations may be provided. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration with adjuvants such as

interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the vaccine of this invention is also advantageous.

The vaccine vectors of this invention may be naked, that is, unassociated with any proteins, adjuvants or other agents which impact on the recipients' immune system. In this case, it is desirable for the vaccine vectors to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline or the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to, calcium ions, may also be used to advantage. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers. Techniques for coating microprojectiles coated with polynucleotide are known in the art and are also useful in connection with this invention.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

EXAMPLES

EXAMPLE 1

Synthetic Gene Construction

Synthetic gene sequences for human papillomavirus proteins L1, E1, E2, and E7 were generated by reverse translation of amino acid sequences using the most frequently used codons found in highly expressed mammalian genes. (R. Lathe, 1985, *J. Mol. Biol.* 183:1-12, which is hereby incorporated by reference). Some adjustments to these codon-optimized sequences were made to introduce or remove restriction sites. Oligonucleotides based on these sequences were chemically synthesized (Midland Certified Reagents; Midland, TX) and assembled by PCR amplification. (J. Haas et. al., 1996, *Current Biology* 6:315-324; and *PCR Protocols*, M. Innis, et al, eds., Academic Press, 1990, both of which are hereby incorporated by reference).

Full-length sequences were cloned into the mammalian expression vector V1Jns (J. Shiver et. al. 1996, in *DNA Vaccines*, eds., M. Liu, et al. N.Y. Acad. Sci., N.Y., 772:198-208, which is hereby incorporated by reference) and sequenced by standard methodology. In cases where the actual sequence differed from the

expected and resulted in amino acid substitution, that sequence was corrected by PCR mutagenesis as previously described (*PCR Protocols*, M. Innis, et al, eds., Academic Press, 1990, pg 177-180).

- Protein expression was evaluated by transient transfection of equal quantities of plasmid DNA into 293 (transformed embryonic human kidney) cells which were harvested at 48 hr post DNA addition. Cell lysates were normalized to provide equal protein loadings. Analysis was by indirect immunofluorescence or immunoblot (Western) analysis using sera prepared to each of the HPV proteins. (Current Protocols in Molecular Biology, eds., F. Ausabel, *et. Al.*, John Wiley and Sons, 1998, which is hereby incorporated by reference).

EXAMPLE 2

Synthesis of HPV 16 L1

- The gene encoding HPV16 L1 was prepared by the annealing and extension of the 14 oligomers listed in FIGURE 17. Five separate extension reactions were performed to create fragments of the gene, designated L1A, L1B, L1C, L1D and L1E by PCR using conditions similar to those described in EXAMPLE 3 and 4, below.

- L1A was constructed using oligomer sequences MN4A1 (SEQ.ID.NO:9), MN4A2 (SEQ.ID.NO:16) and MN4A3 (SEQ.ID.NO:10) which were amplified using the oligomers MN604 (SEQ.ID.NO:32) and MN596 (SEQ.ID.NO:24).

- L1B was constructed using oligomer sequences MN4A4 (SEQ.ID.NO:17), MN4A5 (SEQ.ID.NO:11) and MN4A6 (SEQ.ID.NO:18) and were amplified using the oligomers MN595 (SEQ.ID.NO:23) and MN598 (SEQ.ID.NO:26).

- L1C was created using oligomer sequences MN4A7 (SEQ.ID.NO:12) and MN4A8 (SEQ.ID.NO:19) and were amplified using the oligomers MN597 (SEQ.ID.NO:25) and MN602 (SEQ.ID.NO:30).

L1D was created using oligomer sequences MN4A9 (SEQ.ID.NO:13), MN4A10 (SEQ.ID.NO:20) and MN4A11 (SEQ.ID.NO:14) which were amplified using the oligomers MN597 (SEQ.ID.NO:25) and MN602 (SEQ.ID.NO:30).



L1E was created using oligomer sequences MN4A12 (SEQ.ID.NO:21), MN4A13 (SEQ.ID.NO:15) and MN4A14 (SEQ.ID.NO:22) which were amplified using the oligomers MN601 (SEQ.ID.NO:29) and MN603 (SEQ.ID.NO:31).

- 5 Fragments L1A, L1B, L1C, L1D and L1E resulting from the PCR reactions were gel separated on low melting point agarose with the appropriately-sized products excised and purified using the AgaraseTM method (Boehringer Mannheim Biochemicals) as recommended by the manufacturer. Fragments L1A, L1B and L1C were combined in a subsequent PCR reaction using oligomers MN604 (SEQ.ID.NO:32) and MN600 (SEQ.ID.NO:28) to assemble L1A-B-C; fragments L1D and L1E were assembled to L1D-E by subsequent PCR with the oligomers MN599 (SEQ.ID.NO:27) and MN603 (SEQ.ID.NO:31). The complete gene was then assembled by additional PCR reactions in which fragments L1A-B-C, L1D-E were combined with oligomers MN604 (SEQ.ID.NO:32) and MN603 (SEQ.ID.NO:31) in a final series of PCR reactions. The resulting 1.5 kb product was gel isolated, digested with Bgl II and subcloned into the VIJns and sequenced. In instances where a mutation was observed, it was corrected by PCR mutagenesis as described in EXAMPLE 1. DNA was isolated from a clone with the correct HPV16 L1 DNA sequence and proper orientation within VIJns for use in transient transfection assays as described in EXAMPLE 1.

Transfection Results (HPV16 L1)

- FIGURE 9 shows the HPV16 L1 immunoblot results of lysates of 293 cells transiently-transfected with the VIJns plasmid containing either the native or the codon-optimized, synthetic HPV16 L1. Lanes a, b and c are the expression levels achieved using the synthetic HPV16 L1 expression construct. High levels of immunoreactive material are apparent in each of these lanes with the predominant band at approximately 55 kDa, consistent with the expected molecular weight for full-length HPV16 L1. In contrast, virtually no immunoreactive material is apparent in the lanes containing lysates transfected with the native HPV16 L1/ VIJns plasmid (lanes d, e, and f). Since all cell lysate loadings were normalized and equivalent DNA amounts were used in the transfections, these findings indicate that the synthetic gene sequence greatly increased the levels of HPV16 L1 protein accumulation relative to that of the native gene sequence.

EXAMPLE 3

Synthesis of HPV 16 E1

5 The gene encoding the modified form of HPV16 E1 was assembled from a series of fragments: E1A, E1B, E1C, E1D, E1E and E1F, using the oligomers listed in FIGURE 18. E1A was formed by assembly of oligomers MN605 (SEQ.ID.NO:33), MN606 (SEQ.ID.NO:34) and MN607 (SEQ.ID.NO:35) and amplified using oligomers MN636 (SEQ.ID.NO:64) and MN624 (SEQ.ID.NO:52).

10 E1B was formed by assembly of oligomers MN608 (SEQ.ID.NO:36), MN609 (SEQ.ID.NO:37) and MN610 (SEQ.ID.NO:38) which were amplified with oligomers MN623 (SEQ.ID.NO:51) and MN626 (SEQ.ID.NO:54).

E1C was formed by assembly of oligomers MN611 (SEQ.ID.NO:39) and MN612 (SEQ.ID.NO:40) which were amplified with oligomers MN625 (SEQ.ID.NO:53) and MN628 (SEQ.ID.NO:56).

15 E1D was formed by assembly of oligomers MN613 (SEQ.ID.NO:41), MN614 (SEQ.ID.NO:42) and MN615 (SEQ.ID.NO:43) which were amplified with oligomers MN627 (SEQ.ID.NO:55) and MN630 (SEQ.ID.NO:58).

E1E was formed by assembly of oligomers MN616 (SEQ.ID.NO:44), 20 MN617 (SEQ.ID.NO:45) and MN618 (SEQ.ID.NO:46) which were amplified with oligomers MN629 (SEQ.ID.NO:57) and MN632 (SEQ.ID.NO:60).

E1F was formed by assembly of oligomers MN619 (SEQ.ID.NO:47), MN620 (SEQ.ID.NO:48) and MN621 (SEQ.ID.NO:49) which were amplified with oligomers MN631 (SEQ.ID.NO:59) and MN635 (SEQ.ID.NO:63).

25 Products of these PCR reactions were gel isolated and combined in subsequent rounds of PCR to form a 2 kb gene fragment encoding HPV16 E1 using methods described above. The resulting HPV16 E1 was inserted into the VIJNs expression vector as above and utilized in transient transfection studies as described 30 in EXAMPLE 1.

Transfection Results (HPV16 E1)

FIGURE 10 shows the HPV16 E1 immunoblot results of lysates of 293 cells transiently-transfected with the VIJns plasmid containing either the native, or the codon-optimized, synthetic HPV16 E1. Lanes b and e are the expression levels achieved using the codon-optimized HPV16 E1 expression construct. High levels of HPV16 E1-specific immunostaining are apparent with a predominant band in lanes b and e at 72 kDa, consistent with the expected size for full-length HPV16 E1. In addition, there a number of smaller immunoreactive products which appear to be E1-specific as they are not observed in the mock transfected control (lane c).

A very different expression profile is observed in lysates of cells transfected with the native HPV16 E1/VIJns construct, however. As shown in lanes a and d, only minimal amounts of immunoreactive material can be visualized which is not present in the mock transfection control. Since all cell lysate loadings were normalized and equivalent DNA amounts were used in the transfections these findings indicate that the synthetic gene sequence greatly increased the levels of HPV16 E1 protein accumulation relative to that of the native gene sequence.

EXAMPLE 4

Synthesis of HPV 16 E2

Fragment AD. A 50 μ L reaction containing oligonucleotides 13856-307-2A (SEQ.ID.NO:65), 13856-307-2B (SEQ.ID.NO:71), 13856-307-2C (SEQ.ID.NO:66), and 13856-307-2D (SEQ.ID.NO:72), at 150 nM each, dNTPs 0.5 mM each, Native buffer (Stratagene; La Jolla, CA) and 1 μ L Native Pfu DNA polymerase (Stratagene) was incubated in a GeneAmp 9700 thermocycler (Perkin Elmer Applied Biosystems; Foster City, CA) under the following conditions: 95°C, 2 min.; 20 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 2.5 min. Added to the reaction were primers 13856-307-2PA (SEQ.ID.NO:78) and 13856-307-2PD (SEQ.ID.NO:82) to a final concentration of 400 nM each, and 1 μ L of Native Pfu DNA polymerase. The mixture was incubated for 2 min at 95°C and then 25 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 2.5 min. The gel-isolated full-length fragment AD was amplified for 20 cycles under the same conditions using primers 13856-307-2PA (SEQ.ID.NO:78) and 13856-307-2PD (SEQ.ID.NO:82).

Fragment EH. A 50 μ l reaction containing oligonucleotides 13856-307-2E (SEQ.ID.NO:67), 13856-307-2F (SEQ.ID.NO:73), 13856-307-2G (SEQ.ID.NO:68), and 13856-307-2H (SEQ.ID.NO:74) at 150 nM each, dNTPs 0.5 mM each, Native buffer and 1 μ L Native Pfu DNA polymerase was incubated under the following conditions: 95°C, 2 min; 20 cycles of 95°C, 2 min.; 45 sec.; 55°C, 45 sec.; and 72°C, 2.5 min. Added to the reaction were primers 13856-307-2PE (SEQ.ID.NO:80) and 13856-307-2PH (SEQ.ID. NO:83) to a final concentration of 400 nM each, and 1 μ L of Native Pfu DNA polymerase. The mixture was incubated for 95°C, 2 min.; then 25 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 2.5 min.

Fragment IL. A 50 μ l reaction containing oligonucleotides 13856-307-2I (SEQ.ID.NO:69), 13856-307-2J (SEQ.ID.NO:75), 13856-307-2K (SEQ.ID.NO:70), and 13856-307-2L (SEQ.ID.NO:76) at 150 nM each, dNTPs 0.5 mM each, Native buffer and 1 μ L Native Pfu DNA polymerase was incubated under the following conditions: 95°C, 2 min; 20 cycles of 95°C, 2 min.; 45 sec.; 55°C, 45 sec.; and 72°C, 2.5 min. Added to the reaction were primers 13856-307-2PI (SEQ.ID.NO:81) and 13856-307-2PL (SEQ.ID.NO. 84) to a final concentration of 400 nM each, and 1 μ L of Native Pfu DNA polymerase. The mixture was incubated at 95°C, 2 min.; then 25 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 2.5 min.

Fragment AH. A 50 μ l reaction containing 1.5 μ l each of AD and EH PCR products, dNTPs 0.5 mM each, Native buffer and 1 μ L Native Pfu DNA polymerase was incubated under the following conditions: 95°C, 2 min; 20 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 3.5 min. Added to the reaction were primers 13856-307-2PA (SEQ.ID.NO:78) and 13856-307-2PH (SEQ.ID.NO:83) to a final concentration of 400 nM each, and 1 μ L of Native Pfu DNA polymerase. The mixture was incubated at 95°C, 2 min.; then 25 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 3.5 min.

Fragment IM. A 50 μ l reaction containing 1 μ l of IL PCR product, oligonucleotides 13856-307-2M (SEQ.ID.NO:77) and 13856-307-2PI (SEQ.ID.NO:81) each at a final concentration of 400 nM, dNTPs 0.5 mM each, Native buffer and 1 μ L Native Pfu DNA polymerase was incubated under the following conditions: 95°C, 2 min; 25 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 4 min.

Assembly of AM, full-length HPV16 E2. A 50 μ l reaction containing 1.5 μ l each of fragments AI1 and IM, dNTPs 0.5 mM each, Native buffer and 1 μ L

Native Pfu DNA polymerase was incubated under the following conditions: 95°C, 2 min; 20 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 4 min. Added to the reaction were primers 13856-307-2PA (SEQ.ID.NO:78) and 13856-307-2PM (SEQ.ID.NO:79) at a final concentration of 400 μ M each, and 1 μ L of Native Pfu DNA polymerase. The mixture was incubated at 95°C, 2 min.; then 25 cycles of 95°C, 45 sec.; 55°C, 45 sec.; and 72°C, 4 min. The resultant full-length fragment was isolated by electrophoresis through a 1.2% agarose gel the DNA recovered with a QIAquick column (Qiagen; Santa Clarita, CA) and subcloned into the expression vector VIJns for evaluation.

Transfection Results (HPV16 E2)

FIGURE 11 shows the HPV16 E2 immunoblot results of lysates of 293 cells transiently-transfected with the VIJns plasmid containing either the native, or the synthetic HPV16 E2. Lanes c and d are the expression levels achieved using the codon-optimized HPV16 E2 expression construct. High levels of HPV16 E2-specific immunostaining are visible which appear to be E2-specific as they are not observed in the mock transfected control (lane c).

A very different expression profile is observed in lysates of cells transfected with the native HPV16 E2/VIJns construct, however. As shown in lane e, no immunoreactive material can be visualized. Since all cell lysate loadings were normalized and equivalent DNA amounts were used in the transfections, these findings indicate that the synthetic gene sequence greatly increased the levels of HPV16 E2 protein accumulation relative to those of the native gene sequence.

EXAMPLE 5

Synthesis of HPV 16 E7

The gene encoding HPV16 E7 was assembled from a series of fragments, made using oligomers listed in FIGURE 20.

A 50 μ L reaction containing oligonucleotides 13856-307-7A (SEQ.ID.NO:85), 13856-307-7B (SEQ.ID.NO:87), 13856-307-7C (SEQ.ID.NO:86), and 13856-307-7D (SEQ.ID.NO:88) at 150 nM each, dNTPs 0.5 mM each, Native buffer (Stratagene; La Jolla, CA) and 1 μ L Native Pfu DNA polymerase (Stratagene)

was incubated in a GeneAmp 9700 thermocycler (Perkin Elmer Applied Biosystems; Foster City, CA) under the following conditions: 95°C, 2 min; 20 cycles of 95°C, 45 sec.; 55°C, 45 sec. and 72°C, 2.5 min. Added to the reaction were primers 13856-307-7PA (SEQ.ID.NO:89) and 13856-307-7PD (SEQ.ID.NO:90) to a final concentration of 400 nM, and 1 µL of Native Pfu DNA polymerase. The mixture was incubated for 25 cycles of 95°C, 45 sec.; 55°C, 45 sec. and 72°C, 2.5 min.

The resultant full-length fragment was isolated by electrophoresis through a 1.2% agarose gel in TBE (Current Protocols in Molecular Biology, eds., F. Ausabel, *et. al.*, John Wiley and Sons, 1998, which is hereby incorporated by reference), stained with ethidium bromide, cut from the gel and recovered through a GenElute column (Supelco; Bellefonte, PA) and resuspended in 20 µl water. The sequence was further amplified in a 51 µl reaction containing 2 µl of fragment, 0.5µM each of oligonucleotides 13856-307-7PA (SEQ.ID.NO:89) and 13856-307-7PD, (SEQ.ID.NO:90) dNTPs 0.5mM each, Native buffer and Native Pfu DNA polymerase. The reaction was subjected to 20 cycles of 95°C, 45 sec.; 55°C, 45 sec. and 72°C, 2.5 min. The final amplified product isolated by electrophoresis as described above; the DNA recovered with a QIAquick column (Qiagen; Santa Clarita, CA) and subcloned into VIJns.

20 Transfection Results (HPV16 E7)

FIGURE 12 shows the HPV16 E7 immunoblot results of lysates of 293 cells transiently-transfected with the VIJns plasmid containing either the native (lane e) or synthetic HPV16 E7 (lanes c and d). High levels of HPV16 E7-specific immunostaining are visible in the synthetic HPV16 E7 gene cell lysate lanes which are considerably more intense in appearance than that of the native HPV16 E7 gene cell lysate (lane e). Lanes a and b are negative transfection controls which show the antibody staining is specific to HPV16 E7 sequences. Since all cell lysate loadings were normalized and equivalent DNA amounts were used in the transfections, these findings indicate that the synthetic gene sequence greatly increased the levels of HPV16 E2 protein accumulation relative to those of the native gene sequence.

EXAMPLE 6

Synthesis of the E7 and E2 -encoding genes from HPV6a and HPV18:

- 5 The genes encoding HPV6a E7 and HPV 18 E7 were constructed using similar methods as described in EXAMPLE 4, except that the oligomers used to create the HPV6a E7 and HPV 18 E7 genes contain the sequences listed in FIGURE 21 and FIGURE 22, respectively. The construction of the synthetic genes encoding HPV6a E2 and HPV18 E2 was performed in a similar manner as detailed in
- 10 EXAMPLE 5 using the oligomer sequences listed in FIGURE 23 and FIGURE 24 respectively.

Transfection Results: HPV6a E7 and HPV 18 E7

- FIGURE 13 shows the HPV6a E7 immunoblot results of lysates of 293 cells transiently-transfected with the V1Jns plasmid containing synthetic HPV6a E7 (lanes b and c). High levels of HPV6a E7-specific immunostaining are visible in the region expected for full-length HPV6a E7. A similar profile is found in FIGURE 14 by HPV18 E7 immunoblot analysis of lysates of 293 cells transiently-transfected with the V1Jns plasmid containing synthetic HPV6a E7 (lanes b, c, d and e). High levels
- 15 of HPV18 E7-specific immunostaining are visible where full-length HPV18 E7 would be found as indicated by the location of the purified HPV18 protein control (lane f). There does not appear to be any stained material in the negative control lane a which indicates the staining in the other lanes is HPV18 E7-specific.
- 20

- Expression of the synthetic gene encoding HPV6a E2 in V1Jns was
- 25 evaluated by immunoblot analysis of transfected 293 cells which is shown in FIGURE 15. Lanes a and b are cell lysates of the synthetic HPV6a E2 transfectants; lanes c and d are negative controls. The analogous experiment is shown for HPV18 E2 expression in FIGURE 16. Lanes c and d are the cell lysates of transfections receiving the synthetic HPV18 E2 gene; lanes a and b are the negative controls. Both
- 30 of these figures show measurable levels of E2 product accumulation when the codon-optimized, synthetic gene is expressed in mammalian cells.

These results indicate that the synthetic gene rebuilding is not limited to HPV16 genes. Rather, codon optimization of other HPV types also permits significant levels of E7 and E2 product accumulation in mammalian cells.

EXAMPLE 7

Construction of replication-defective FG-Ad expressing HPV antigen

5 Starting vectors

Shuttle vector pHCMVIBGHpA1 contains Ad5 sequences from bp1 to bp 341 and bp 3534 to bp 5798 with a expression cassette containing human cytomegalovirus (HCMV) promoter plus intron A and bovine growth hormone polyadenylation signal.

- 10 The adenoviral backbone vector pAdE1-E3- (also named as pHVad1) contains all Ad5 sequences except those nucleotides encompassing the E1 and E3 region.

Construction of Ad5. HPV16 E2

- 15 1. Construction of adenoviral shuttle plasmid pA1-CMV1-HPV16 E2 containing HPV16 E2 under the control of human CMV promoter and intron A.

The HPV16 E2 insert was excised from pVIJNS-HPV16 E2 by restriction enzyme Bgl II, EcoRI and then cloned into Bgl II, EcoRI digested shuttle vector pHCMVIBGHpA1.

- 20 2. Homologous recombination to generate plasmid form of recombinant adenoviral vector pAd-CMV1-HPV16 E2 containing HPV16 E2 expression cassette.

- Shuttle plasmid pA1-CMV1- HPV16 E2 was digested with restriction enzymes BstZ17 and SgrA1 and then co-transformed into *E. coli* strain BJ5183 with linearized (ClaI digested) adenoviral backbone plasmid pAdE1-E3-. A colony was verified by PCR analysis. The vector was transformed to competent *E. coli* HB101 for large quantity production of the plasmid.
- 25

3. Generation of recombinant adenovirus Ad.CMV1- HPV16 E2 in 293 cells.

- 30 The pAd plasmid was linearized by restriction enzyme PacI and transfected to 293 cells using CaPO₄ method (Invitrogen kit). Ten days later, 10 plaques were picked and grown in 293 cells in 35-mm plates. PCR analysis of the adenoviral DNA showed virus were positive for HPV16 E2.

4. Evaluation of large scale recombinant adenovirus Ad.CMV1- HPV16 E2

A selected clone was grown into large quantities through multiple rounds of amplification in 293 cells. Expression of HPV16 E2 was also verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus. The recombinant adenovirus was used for evaluation in mice and rhesus monkeys.

Method of Treatment

- A person in need of therapeutic or prophylactic immunization against infection with human papillomavirus virus is injected with HPV DNA encoding all or part of; HPV L1, E1, E2, E4 or E7 and combinations thereof. The injection may be i.p., subcutaneous, intramuscular or intradermal. The HPV DNA may be used as a primer of the immune response or may be used as a booster of the immune response. The injection of DNA may antedate, coincide or follow injection of the person with a pharmaceutical composition comprising HPV virus like particles (containing only L1 protein or containing both L1 and L2 proteins, or containing mutant forms of one or more proteins), capsomeres, inactivated HPV, attenuated HPV, compositions comprising HPV-derived proteins, or combinations thereof.

EXAMPLE 8

The use of a synthetically-expressed HPV E protein as a model tumor antigen.

Generation of a tumor cell line that expresses HPV 16 E2.

- A Not I-Hind III restriction digest fragment containing the synthetic coding sequence for HPV 16 E2 (see above) was ligated with Not I, Hind III digested expression vector pBJ/neo/CCR2B which has a neomycin resistance marker and drives the expression of the transgene with the HCMV immediate early promoter. The resultant plasmid, pBJ-16 E2, was characterized by restriction digestion, sequence analysis of the cloning junctions, and the ability to induce E2 protein expression in transiently-transfected A293 or CT26 cells. A stable cell line was generated transfection of CT26 cells using Lipofectamine (Gibco BRL). CT26 cells, a fully-transformed line derived from a BALB/c mouse colon carcinoma, have been widely used to present model tumor antigens. (Brattain et al., 1980 *Cancer Research*

40:2142-2146; Fearon, E. et al., 1988 *Cancer Research*, 48:2975-2980; both of which are incorporated by reference).

After 48 hours, cells were trypsinized, diluted 1:10, 1:100, 1:1000 or 1:10000 into medium and plated in 100mm² plates. After 24 hours, the medium was replaced with selection medium containing 400µg/mL G418. After two to three weeks, well-isolated colonies of cells were recovered using cloning rings and transferred to 48-well plates. One clone was positive for E2 expression by immunoblot analysis and was subjected to two further rounds of cloning by limiting dilution. One G418 resistant, E2-positive clonal isolate was used to establish the cell line JCL-031. (FIGURE 25).

When inoculated into (syngeneic) BALB/c mice by subcutaneous injection, JCL-031 cell induced tumors with the kinetics similar to those as the parental CT26 line. Cells cultured from recovered tumors were G418 resistant and expressed E2.

Induction of immunity in mice by immunization with VIJns:E2 DNA.

BALB/c mice were immunized multiple times by intramuscular injection with the DNA VIJns:16E2. Spleens from two randomly-chosen mice in each dose group were pooled, splenocytes prepared, and assayed in an murine interferon gamma Elispot assay. (Lalvani et al. 1997 *J. Exp. Med.* 186: 859-865; Forsthuber, T., et al 1996 *Science* 271: 1728-1730; Chu, R. et al. 1997. *J. Exp. Med.* 186: 1623-1631, each of which is incorporated by reference.) Splenocyte cultures were incubated at 37°C for 24 hr. in the presence of a pool of 36 overlapping 20 amino acid residue peptides (final concentration, 4µg/mL each) scanning the full length of HPV 16 E2. Interferon gamma was captured on the substrate by monoclonal antibody (mAb) R4-6A2 (Pharmagin), and detected with biotinylated mAb XMGI.2 (Pharmagin) and a streptavidin-alkaline phosphatase conjugate (Pharmagin). Results are shown in Table A, below. The immunized mice developed CD4⁺ immune responses to HPV (Table A, below).

Immunization with E2 DNA did not induce detectable anti-E2 antibody responses.

5

Table A

Dose Group	Immunization	E2-specific spots (per 10^6 cells)
1	E2 DNA 1	392
2	E2 DNA 2	96
3	E2 DNA 3	134
4	Control DNA 1	0
5	Control DNA 2	2

Protection from challenge with JCL-031 cells.

BALB/c mice, immunized with VJNs: E2 DNA, or control DNA, were challenged by subcutaneous injection of 5×10^5 JCL-031 cells into the left inguinal region. Tumor growth was monitored by palpation or caliper measurement for a four-week period. FIGURE 26 reports the fraction of each dose group that remained tumor free. The group that had been immunized with an E2-expressing plasmid was significantly protected from tumor development compared to the control group.

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